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ORIGINAL PAPER

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Physiology of biofilms of thermophilic bacilli—potential consequences for cleaning

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Abstract Thermophilic *Bacillus* species readily attached and grew on stainless steel surfaces, forming mature biofilms of $>10^{6.0}$ cells/cm^2 in 6 h on a surface inoculated with the bacteria. Clean stainless steel exposed only to pasteurized skim milk at 55 °C developed a mature biofilm of $>10^{6.0}$ cells/cm^2 within 18 h. When bacilli were inoculated onto the steel coupons, 18-h biofilms were 30 μm thick. Biofilm growth followed a repeatable pattern, with a reduction in the numbers of bacteria on the surface occurring after 30 h, followed by a recovery. This reduction in numbers was associated with the production of a substance that inhibited the growth of the bacteria. Variations in the environment, including pH and molarity, affected the viability of the cells. Chemicals that attack the polysaccharide matrix of the biofilm were particularly effective in killing and removing cells from the biofilm, demonstrating the importance of polysaccharides in the persistence of these biofilms. Treatment of either the biofilm or a clean stainless steel surface with lysozyme killed biofilm cells and prevented the attachment of any bacteria exposed to the surface. This suggests that lysozyme may have potential as an alternative control method for biofilms of these bacteria.

Keywords Biofilms · Cleaning · Thermophilic bacilli · Dairy industry

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Introduction

Milk can be a source of bacteria or spores that later germinate into bacteria [19]. Such bacteria may attach to the internal surfaces of processing equipment in dairy manufacturing plants and grow in selective environments. Thermophilic bacilli such as *Geobacillus stearothermophilus* grow in the evaporators of milk powder manufacturing plants in areas where the bacteria may adhere to the surfaces of the manufacturing plant and temperatures are approximately 55 °C [22]. These bacteria produce exopolysaccharides and multiply, with more cells thereby attaching to the exopolysaccharide matrix to generate complex communities called biofilms [30]. Although bacteria of more than one species co-exist as biofilms with greater metabolic advantages than single species in most natural aquatic systems, single-species biofilms tend to colonize artificial substrates, such as medical implants and equipment surfaces in dairy manufacturing plants [24].

Biofilms have a dynamic and complex architecture that is now being recognized as having significance in many environments [9]. The formation of biofilms depends on the substrate properties, nutrient availability and flow hydrodynamics [6], although the architecture of the biofilm and the polysaccharide composition are probably unique features of the environment in which the biofilm develops [30]. Biofilms are a survival mechanism, protecting and propagating the bacteria in adverse conditions, such as turbulent flows or nutrient limitation [11]. Bacteria in biofilms are much more resistant to biocides and cleaning agents than their free-living counterparts [10]. This is attributed to factors such as limited diffusion or neutralization of biocides through the exopolymer matrix, a lower rate of growth due to nutrient limitation and acquisition of new genetic traits, such as resistance-conferring genes [11], resulting in the formation of chemical-resistant phenotypes in biofilms [1].

Studies on biofilms in dairy manufacturing plants have focused on *Pseudomonas* species and food-borne pathogens [12]. There are few publications dealing with the spore-forming bacteria [17] and only limited information on thermophilic spore-forming bacilli as biofilms in dairy manufacturing plants [25].

Cleaning dairy manufacturing plants to remove biofilms is important to maintain product quality and to reduce energy loss that may occur through heavy biofilm colonization. Most cleaning-in-place (CIP) systems used in the industry are designed and optimized in terms of food product and soil removal [8]. Thus, CIP focuses on cleaning plant surfaces and disinfection to reduce the viability of the bacteria remaining after cleaning. Such procedures do not necessarily reduce the likelihood of biofilm development, nor do they necessarily remove established biofilms. To do this, more efficient CIP procedures are required that target both biofilm and food product.

More novel approaches to controlling biofilms include substrate modification, biological control or specially formulated cleaners. Substrates with altered properties, such as hydrophobicity or surface roughness, are less susceptible to bacterial attachment [3], while those impregnated with antimicrobial agents may kill attached bacteria, preventing their metamorphosis into a biofilm. Special cleaners currently used include biological enzymatic mixes of proteases and carbohydrases [18].

The aim of this study was to learn about the factors affecting the growth of biofilms of thermophilic bacilli isolated from the dairy industry by investigating the biofilm growth cycle, the effect of environmental factors on biofilm growth, the effect of chemical treatments on the biofilm and the use of surface treatments to control biofilms of thermophilic bacilli on stainless steel.

Materials and methods

The organism and planktonic culture preparation

Bacillus flavothermus strain B12-C^{m} stock culture was maintained at −20 °C, using Microbank cryobeads (Pro-Lab Diagnostics, Austin, Tex.) at the Biofilm Research Unit, Institute of Food, Nutrition and Human Health (Massey University, Palmerston North, New Zealand). The cryobeads were inoculated into 10 ml of trypticase soy broth (TSB; Becton Dickinson, Cockeysville, Md.) at 55 °C for 12 h. This seed culture (100 µl) was used to inoculate 200 ml of TSB in a 500-ml Duran bottle which was incubated at 55 °C with agitation for 6 h. These planktonic cultures were centrifuged at 3,000 *g* for 10 min; and the cell pellet was then washed and resuspended in sterile distilled water. These cells were used to inoculate the biofilm reactor as described below.

Substrate, milk and chemicals

Stainless steel (grade 316 with a 2b finish) was cut into coupons and cleaned as reported previously [15] for the laboratory reactor type I. We also used similarly cut and cleaned coupons of electropolished stainless steel for studying how biofilms grew on a different substrate. Ugi grade (highly hygienic) steel (from Dr. B. Webster, Industrial Research Ltd., Wellington, New Zealand) was similarly cleaned and used for reattachment studies with these cells.

Pasteurized skim milk (0.05% fat) was used for all biofilm reactor studies.

All the chemicals and biochemicals used were of analytical grade and were purchased locally. The lysozyme (chicken egg white) used was from Sigma Chemical Co., St. Louis, Mo.

Biofilm generation

A laboratory reactor (reactor I) was used to generate 18-h mature biofilms on the coupons [15]. Cleaned and sterile stainless steel coupons inserted into clean silicone tubing were the substrates for biofilm formation. The entire system was cleaned and autoclaved before use. The coupons were inoculated with washed B12-C^{m} cells by flooding the tubing containing the coupons with a 6-h culture and incubating them at ambient temperature for 30 min. Pasteurized skim milk at 55 °C was recirculated past the coupons at 140 ml/min for up to18 h to generate biofilms. Fresh milk was continuously added to the system (working volume 225 ml) at 4.7 ml/min and the surplus overflowed to drain. This dilution rate exceeded the growth rate of the bacteria and avoided any increase in the numbers of planktonic cells. The coupons with biofilms attached to them were aseptically cut from the tubing and rinsed with sterile distilled water prior to examination for biofilms. Fresh, uninoculated, clean and sterile coupons were used in two experiments to estimate the time taken for biofilms to form without any inoculation.

Examination of biofilm cells

Biofilm cells were examined for total cell numbers using direct epifluorescence microscopy (DEM), after staining with acridine orange [25]. Viable cell numbers were determined by impedance using the Bactrac 4100 micro-organism growth analyser [14]. Ten colonies from biofilm cells grown on trypticase soy agar (TSA; Becton Dickinson) were confirmed as belonging to the same strain of *B. flavothermus* by randomly amplified polymorphic DNA (RAPD) analysis [27].

Long-term biofilms

To study the growth of a mature biofilm, the biofilm reactor was maintained for up to 8 days. Sections of tubing containing coupons with a biofilm were removed and rinsed with sterile distilled water at 22 °C. The viable cell numbers were again counted using the Bactrac 4100; and the coupons were also examined for total count using DEM. Confocal scanning laser microscopy (CSLM) was used on the same acridine orange-stained samples to observe the structure of the biofilms formed at different times.

Demonstration of bacteriocin-like inhibitory substance activity

The coupons and the milk surplus overflow (“milk-out”) were aseptically removed from the biofilm reactor at various stages of the biofilm growth. At each given time, duplicate samples of the coupon and 100 µl of the milk-out on a sterile filter paper disc were placed on TSA plates freshly spread with 100 µl of a 6-h culture of *B. flavothermus* B12-C^{m} cells. The plates were then incubated at 55 °C for 12 h. Inhibitory activity was seen as a zone of no growth around the coupon or disc.

Biofilm removal and inactivation studies

In order to study the effect of different environmental treatments on the stability, removal or inactivation of the biofilm, coupons containing a biofilm were subjected to various treatments (Table 1),

Table 1 Treatments given to 18-h biofilms of *Bacillus flavothermus* on stainless steel coupons

Treatment type	Application details
Mechanical	Agitation (vortex mixing) in sterile water for 1 min Scrubbing with Pyroneg cleaner
Physicochemical	Phosphate buffer pH 4–10, 30 min, 22 °C NaCl solution 0.5–1.0 M, 30 min, 22 °C
Polysaccharide removal	50 mM sodium metaperiodate, 60 min, 22 °C 10% trichloroacetic acid (TCA), 15 min, 100 °C 2% lysozyme, 60 min, 37 °C [25]
Protein solubilization/degradation	2% sodium dodecyl sulphate (SDS) 10 min, 100 °C 2% trypsin, 3 h, 37 °C [25]
Pro-oxidants	Potassium monopersulphate (1 mg/ml) in saline, 30 min 22 °C, followed by sodium thiosulphate (9 mg/ml), 5 min, 22 °C [33]
Antibacterial agent	Nisin (1 mg/ml) in 0.02 M HCl [5]

based upon previous work in our laboratory and other studies [4, 5, 10,12, 13, 25, 33]. Following treatment, the coupons were rinsed five times with sterile distilled water at 22 °C. The number of viable cells and the total number of cells were expressed as a percentage of the control value.

Reduced attachment and prevention of biofilm growth

The attachment and the growth of bacteria on stainless steel with a 316 2b finish, electropolished 316 and Ugi grade (attachment only) were compared.

To examine the effectiveness of lysozyme in removing biofilms and preventing the reattachment of cells, coupons containing a biofilm were cleaned with lysozyme (0.5, 1.0, 2.0%), trypsin (1%), or lysozyme followed by tryptic digestion. The cleaned coupons were then rinsed with five washes of sterile distilled water to remove the enzymes. The coupons were then exposed to a 6-h culture of washed *B. flavothermus* B12-C^m cells for 30 min and rinsed to remove loosely attached cells. The number of viable attached cells was determined using impedance. Biofilms on coupons were killed by boiling at 100 °C for 15 min or by exposure to 0.4% formaldehyde for 15 min. The killed biofilms and clean stainless steel coupons were used as control preparations. The presence of lysozyme on the stainless steel surface was checked by immunolabelling, using anti-lysozyme antibody followed by fluorescein-labelled anti-mouse immunoglobulin.

To examine the effectiveness of lysozyme and other proteins in preventing the attachment of cells to clean stainless steel and biofilm formation, sterile coupons treated with distilled water were compared with coupons treated with 2% solutions of bovine serum albumin (BSA), trypsin or lysozyme for 30 min at ambient temperature. The coupons were then rinsed thoroughly with five washes of sterile distilled water and inserted into different sterile reactor tubings. For these trials, the milk made a single pass through the reactor, to avoid the possibility of recirculated bacteria influencing the results. The reactor was not inoculated with cells; and a natural biofilm from the milk was allowed to grow over 18 h. The coupons were then removed aseptically and the viable count was estimated by impedance monitoring.

Results

Biofilm growth

Biofilm development in a reactor containing coupons inoculated with *B. flavothermus* B12-C^m culture produced typical levels of $>10^6$ cells/cm^2. RAPD analysis confirmed that this strain, and not some adventitious strain from the milk, predominated in the biofilm. However, even when sterile coupons were used, biofilm development (natural biofilm) from low levels ($<10^0$ cell/mL) of bacteria in the pasteurized milk still occurred within 18 h, with viable counts $>10^6$ cells/cm^2.

Biofilm cycle

In long-term reactors, the number of viable cells varied with the sampling time. DEM examination revealed a continuous biofilm at the end of 18 h. There was a decrease by 10^2 cells/cm^2 at 30 h, followed by a recovery and an increase in cell numbers (Fig. 1). Microscopic examination of 30-h coupons showed few bacilli; and some sites showed that bacilli had been removed from the biofilm, leaving behind impressions or ″footprints″ presumably consisting of residual glycocalyx [23]. CSLM showed that 18-h biofilms had a maximum thickness of 30 μm. Cells forming the recovered biofilm were confirmed to be B12-C^m by RAPD analysis.

Production of inhibitory substances by the biofilm

The B12-C^m bacteria remaining on the coupons and those from the milk-out of the reactor, sampled at 30 h of growth at 55 °C, produced a zone of inhibition on the TSA plate harbouring the lawn of planktonic B12-C^m cells from a 6-h laboratory culture (Fig. 2). This zone of inhibition was not evident when the lawn was prepared with B12-C^m bacteria from the milk-out itself, nor was it

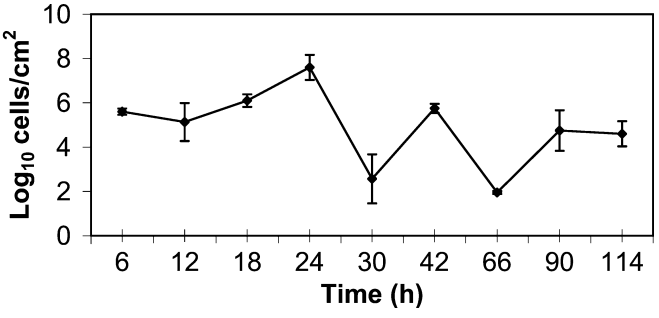

Fig. 1 Patterns in biofilm growth represented by a reduction in cell numbers. *Error bars* indicate standard deviation for duplicate tests (two independent trials)

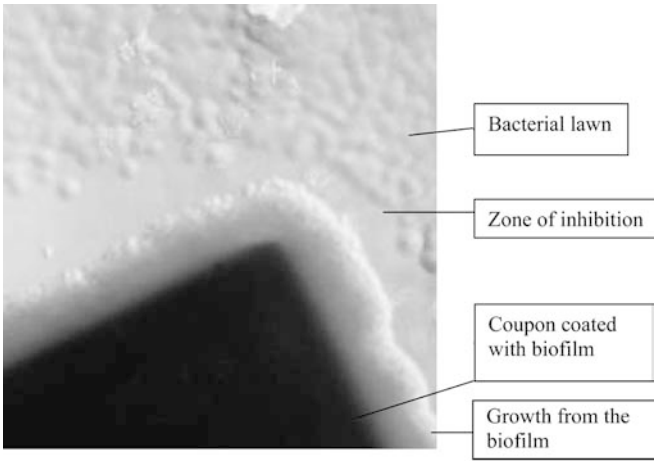

Fig. 2 Zone of inhibition surrounding a biofilm on a bacterial lawn. Note the growth of bacteria from the biofilm around the edge of the coupon, outside of which is a cell-free zone and then a bacterial lawn

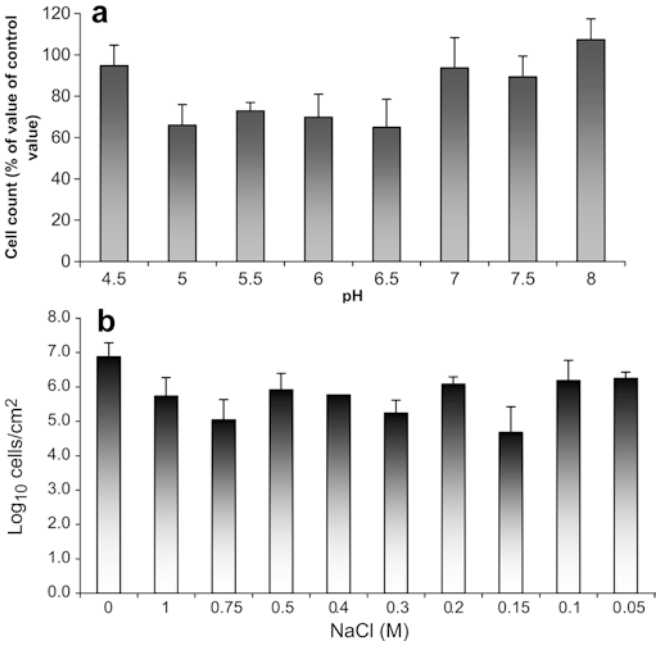

Fig. 3a, b Stability of biofilms. **a** The effect of pH on the stability of a biofilm. Stainless steel coupons with an 18-h *Bacillus flavothermus* B12-C^m biofilm were incubated for 30 min with sterile distilled water (control) or phosphate buffers ranging from pH 4.5 to pH 8.0, then rinsed five times with distilled water and their bacterial count estimated by impedance monitoring. *Error bars* indicate standard deviation on duplicate tests. **b** The effect of ionic strength on the stability of a biofilm. Stainless steel coupons with an 18-h *B. flavothermus* B12-C^m biofilms were incubated for 30 min with sterile distilled water (control) or saline solutions of different molarities, then rinsed five times with distilled water and their bacterial count estimated by impedance monitoring. Duplicate biofilms were tested with each treatment. *Error bars* indicate standard deviation on duplicate tests

seen with coupons taken at earlier times (6, 12, 18, 24 h). Bacterial growth originating from the biofilm around the stainless steel coupon suggests these cells are actively producing an inhibitory substance rather than a pH effect. The biofilm B12-C^m bacteria inoculated onto TSA by touching the surface with a stainless steel coupon were also able to inhibit planktonic growth, while planktonic bacteria cells grew unaffected around sterile stainless steel coupons, indicating that the stainless steel itself had no role in this inhibitory activity.

Biofilm removal and inactivation studies

Agitating the coupons in a test tube on a standard vortex mixer at full speed for 1 min at room temperature decreased the number of viable cells in the biofilm on the stainless steel coupons from 10^6 cells/cm^2 to 10^4 cells/cm^2. Cleaning by scrubbing the biofilm-containing coupons was highly successful in removing the biofilm, producing a surface visually clean under DEM and with $< 10^0$ viable cell/cm^2, using the Bactrac 4100.

To determine the effect of environmental conditions on the stability of the biofilm, the viability of biofilm cells on coupons soaked in phosphate buffers of differing pH was compared with that of biofilm cells on coupons soaked in distilled water (control). Biofilm cells on coupons kept at pH 6.5 had the least viability, the viable cell number being just half that of the control (Fig. 3a). DEM revealed that the confluent biofilm seen at pH 7.5 appeared to have disintegrated at pH 6.5, with fewer cells adhering to the substrate. Incubating the biofilms with saline of different molarities (Fig. 3b) showed that saline at 0.15 M was the most inhibitory to biofilm viability, causing disintegration of the biofilm structure.

Evidence that chemicals that degrade polysaccharide affected the stability of the biofilm was shown by treatment with sodium metaperiodate, trichloroacetic acid (TCA) and lysozyme. Both metaperiodate and TCA treatments caused a 100% loss of viability of the 18-h *B. flavothermus* B12-C^m biofilm (Fig. 4) but, in terms of total cell numbers, averages of 8% and 28% of the control value were seen on the coupon surface, using DEM. This shows that the cells had been killed, although many cells remained on the surface. In both cases, the cells that were present were linked with their polysaccharide matrix, whereas the cell-free areas were devoid of polysaccharides. Lysozyme was capable of 100% efficiency in killing biofilm cells at concentrations as low as 0.1% whereas at lower concentrations, such as 0.05% and 0.001%, there was no loss of biofilm viability. Although 2% lysozyme resulted in a 10^7 cells/cm^2 reduction in the numbers of the cells and removal of all of the polysaccharide matrix from the coupon surface (confirmed visually), an increasing amount of cells in a biofilm were left behind at lower concentrations.

Treatment with an anionic detergent sodium dodecyl sulphate (SDS) and the protease trypsin was aimed at determining the effect of agents affecting protein on the stability of the biofilm. Boiling biofilms with 2% SDS for 10 min reduced cell viability by 80%, allowing only spores to survive. Only 2% of the total cells were

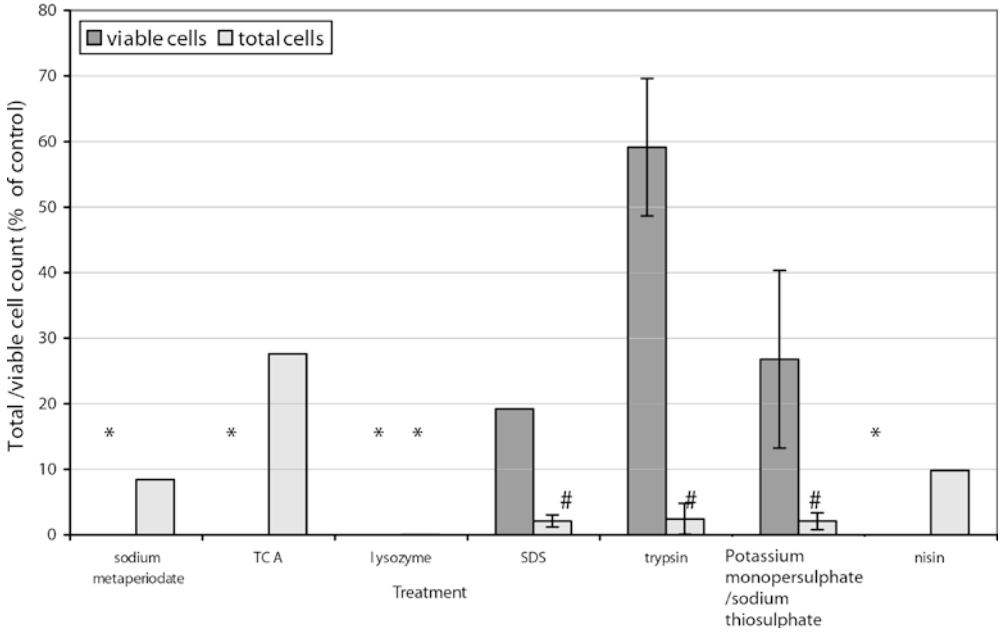

Fig. 4 Effect of various chemicals on the total count and number of viable cells in biofilms of *B. flavothermus* B12-C^{m}. *Error bars* show standard deviation on duplicate trials. The 18-h biofilms on at least two different stainless steel coupons were treated with 50 mM sodium metaperiodate, 10% trichloroacetic acid (*TCA*), 2% lysozyme, 2% sodium dodecyl sulphate (*SDS*), 2% trypsin, 1 mg potassium monopersulphate/ml followed by 9 mg sodium thiosulphate/ml, or 1 mg nisin/ml. The coupons were then rinsed five times in sterile distilled water and the number of viable cells was counted by impedance monitoring. Each total count is an average obtained by counting five different fields of acridine orange-stained fluorescent cells under the microscope. Baseline values of viable cells in a biofilm (water-washed biofilm samples) were $10^{7.0 \pm 0.57}$ cells/cm^2. The total count by direct epifluorescence microscopy (DEM) averaged at 2,080 cells/field. *At points marked with asterisks*, no cells were detected. For the SDS, trypsin and potassium monopersulphate/sodium thiosulphate treatments (*hash-marks*), cells were difficult to visualize under DEM and the total counts may therefore underestimate residual cell numbers

observed using DEM, demonstrating incomplete removal of the vegetative cells. Trypsin was only 50% successful in killing biofilm cells and left 2% of the total cells on the coupon surface. In both cases, the polysaccharide matrix was seen, but the cells were thin and difficult to visualize and count.

The pro-oxidant potassium monopersulphate, at a concentration of 1 mg/ml, killed the biofilm cells; but again 2% of the cells were not removed from the surface. The antibiotic nisin at 1 mg/ml killed 100% of the biofilm; but up to 10% of the total biofilm cells, which appeared to be swollen with ruptured cell walls, were detected on the surface.

Controlling bacterial attachment and biofilm development

The extent of attachment of the bacteria to different stainless steel surfaces (electropolished, Ugi, control 316) varied. Most attachment occurred to the control steel ($10^{1.6}$ cells/cm^2), with similar attachment to the electropolished steel ($10^{1.5}$ cells/cm^2; $P > 0.5$). Attachment was significantly reduced when using Ugi steel ($10^{0.9}$ cells/cm^2; $P < 0.001$).

Biofilm reactors with coupons made of electropolished stainless steel generated biofilms in 18 h, with counts averaging $10^{5.43 \pm 0.08}$ cells/cm^2 and $10^{5.34 \pm 0.37}$ cells/cm^2 when using uninoculated and *B. flavothermus*-inoculated coupons, respectively. Note that the development of biofilms on Ugi stainless steel was not examined.

When the biofilm-containing coupons were cleaned with lysozyme at $>0.1\%$ concentration, there was no reattachment of cells after exposing them to a suspension of *B. flavothermus* B12-C^{m} cells for 30 min (Fig. 5). This time period was chosen for reattachment studies as this is the average time taken for irreversible attachment of cells to the surface. Shorter times may lead to underestimates of cell numbers (reversibly attached bacteria may detach) and longer times may lead to overestimates of cell numbers, as the generation time of *B. flavothermus* is about 30 min. When lysozyme treatment of the biofilm was followed by trypsin treatment to remove the lysozyme, reattachment occurred. Up to $10^{3.33}$ cells/cm^2 of *B. flavothermus* B12-C^{m} also attached to formaldehyde- or heat-killed biofilm cells, indicating that the presence of cell material did not prevent the further attachment of more bacteria.

Lysozyme treatment of clean coupons also inhibited the attachment of *B. flavothermus* B12-C^{m} cells and was dose-dependent (Fig. 5). No short-term attachment was seen on stainless steel treated with 2% lysozyme, whereas the extent of reattachment increased with decreasing concentration of lysozyme, with maximum reattachment at 0.1%. Prevention of attachment and

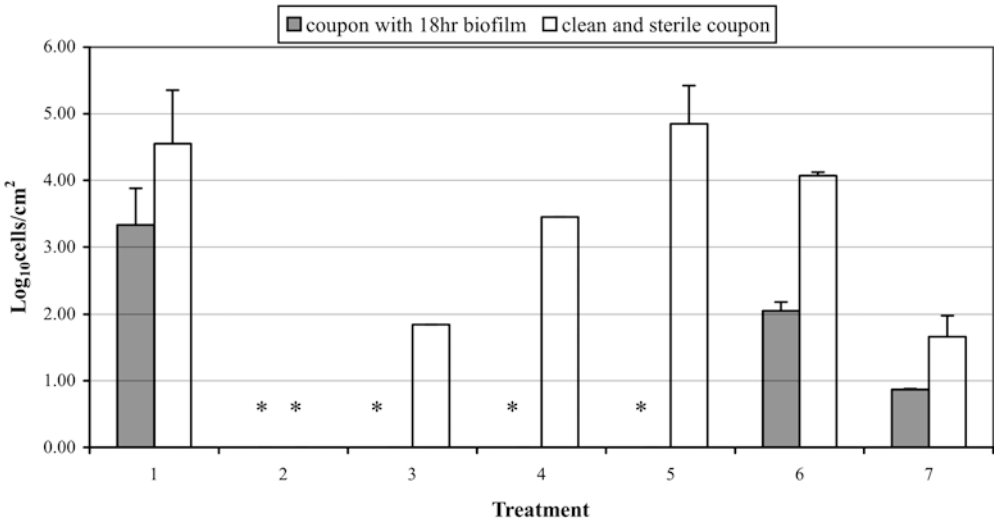

Fig. 5 Effect of lysozyme treatment on the reattachment of thermophilic bacilli to stainless steel either with clean stainless steel or an 18-h biofilm of strain B12-C^m at the start of the experiment. *Error bars* show the standard deviation of duplicate trials. The stainless steel coupons, either clean and sterile or containing an 18-h biofilm of strain B12-C^m, were rinsed to remove loosely attached cells, treated with various agents and then washed five times with sterile distilled water. The coupons were subsequently incubated for 30 min at ambient temperature with a B12-C^m cell suspension. They were then rinsed and the number of cells attached to the coupons was counted by impedance monitoring. The control (*column 1*) used biofilms killed with boiling or 0.4% formaldehyde for 15 min. In the other cases, the 18-h viable biofilms were treated with 2% lysozyme (*column 2*), 1% lysozyme (*column 3*), 0.5% lysozyme (*column 4*), 0.1% lysozyme (*column 5*), 2% trypsin (*column 6*) or 0.1% lysozyme followed by 2% trypsin (*column 7*). The values are averages of the results from two independent experiments. *At points marked with asterisks*, no viable bacteria were detected on the coupons

biofilm development following treatment of stainless steel coupons with 1% lysozyme was maintained for at least 8 h. Trypsin pretreatment could not prevent attachment of cells, but further treatment with lysozyme decreased the reattachment by approximately $10^{0.5}$ cells/cm^2. Immunolabelling studies revealed the presence of fluorescent conjugates of immunoglobulin/anti-lysozyme antibodies on lysozyme-treated coupons with or without biofilm when observed using DEM. This label was lost when the lysozyme treatment was followed by trypsin treatment.

BSA and trypsin reduced the development of biofilms over 18 h on treated coupons to $10^{2.0}$ cells/cm^2 and $10^{2.3}$ cells/cm^2, respectively, compared with the biofilm viable count of $10^{6.0}$ cells/cm^2 on untreated coupons (Fig. 6). However, pretreatment with lysozyme prevented any biofilm formation. On microscopic observation of the coupons, it was found that there was a confluent biofilm on the uncoated control, with small patches of biofilm on the trypsin-coated coupons and more such patches on the BSA-coated coupons. On the lysozyme-coated coupons, less than 10^0 cell/cm^2 was counted per five fields.

Discussion

Biofilm formation

Stainless steel coupons cut from new material, cleaned and sterilized, were susceptible to biofilm formation and showed colonization from pasteurized skim milk within 6 h and mature biofilms in 18 h. The rapid growth of biofilm on new, totally clean stainless steel implies that, although cleaning food-contact surfaces is important, this cannot control bacterial colonization and preventing attachment of bacteria to control biofilm development is therefore important.

Biofilms (18 h) of strain B12-C^m were revealed by CSLM to be approximately 30 μm thick and consisted of a single species of *Bacillus*, as determined by molecular fingerprinting using RAPD analysis. This was important, as it showed that no other bacteria from the milk supply colonized the surfaces during these experiments.

Biofilm, once formed, remains bound to the substrate in a very dynamic equilibrium for long periods of time, depending on nutrient availability, cleaning procedures, etc. Some studies have used long-term biofilms for cleaning-evaluation experiments, such as 4-day biofilms of *P. aeruginosa* on stainless steel [16].

Further work is needed to determine whether the phenomenon of fluctuating cell numbers in the biofilm over time, as observed in this study, occurs with other bacteria. Ludensky [21] found a fluctuating pattern in biofilms examined using a heat transfer flux-measuring system. This phenomenon has implications for the release of slugs of contamination into an industrial system such as a food manufacturing plant.

These biofilm fluctuations may be a response to stress in the biofilm community as cell density increases to a point where the cells are unable to obtain sufficient requirements for survival. The release of biofilm cells is presumably triggered by quorum sensing and, if fully understood, may lead to a new method of controlling

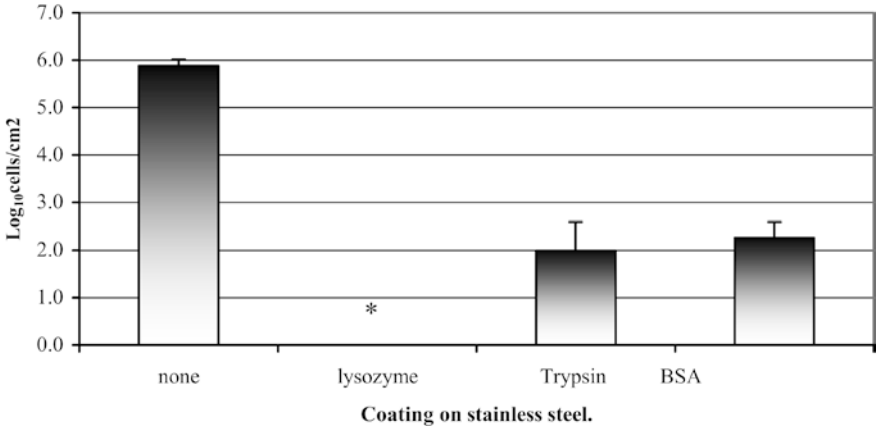

Fig. 6 The effect of precoating stainless steel on biofilm formation. Stainless steel coupons were coated with a 2% solution of lysozyme, trypsin or BSA and were then inserted into different reactor tubings together with untreated sterile coupons and run for 18 h with pasteurized skim milk without inoculation. The coupons were then removed and cells were counted by impedance monitoring. In the case of lysozyme treatment, *marked with an asterisk*, no viable bacteria were detected. *Error bars* indicate the standard deviation on duplicate tests

biofilms. In this instance, the variation appeared to correlate with the production of inhibitory activity.

Influence of physicochemical and biochemical factors on biofilm structure, viability and detachment

Stoodley et al. [29] showed the influence of the flow rate of the liquid passing over the biofilm on the structure of biofilms. However, many factors besides flow rate influence biofilm removal. Biofilms are tightly bound to the surface, but agitation removes a lot of the loosely attached cells. This would influence any cleaning regime, as high-velocity flushing could decrease the microbial load and therefore make cleaning more efficient. Understandably, mechanical scrubbing of the surface removed all attached biomaterial in this small area. However, mechanical scrubbing of the surfaces of a large-scale, enclosed manufacturing plant is impossible. A study performed at an industrial scale showed that mechanical scrubbing and high-pressure sprays were only partially successful in the removal of biofilms [16]. The sprays were of limited use and resulted in the formation of aerosols and frothing of the cleaners, both of which were undesirable.

Physicochemical factors such as a pH of 6.5 helped in the detachment of biofilms. This may have been due to maximum release of proteases or polysaccharidases at this pH. This parallels reports that attachment of certain planktonic bacilli is enhanced at pH 4.0 and pH 10.0, compared with that at pH 7.0 [20]. The same report noted increased lipolytic activity at pH 4.0 and pH 10.0 [20]. Some thermophilic bacilli are known to be prolific producers of thermostable proteinases [26] that are secreted into the extracellular medium. Such proteinases may cause the breakdown of cell surface proteins. Cell surface proteins are responsible for the initial attachment of these bacteria to substrates [25]. This proteolytic activity is likely to be affected by pH and this could explain the observed differences in detachment at different pHs.

Influence of substrate and biological pretreatment on biofilm formation

Biofilms on electropolished stainless steel did not fully cover the stainless steel substrate, nor did they appear as thick under DEM as those on control 316 stainless steel; but the difference was not great. The lower number of cells on electropolished stainless steel may be due to the special treatment given to the stainless steel, which alters its electrical charge.

Trypsin and BSA, like the milk proteins previously studied [2, 25], adsorb onto steel, blocking some sites of cell attachment and thereby inhibiting initial cell attachment. Trypsin, besides its blocking effect, could also cause proteolysis of some protein surface-attachment factors, explaining the slightly lower biofilm growth here. However, the lower numbers of cells attached continued to proliferate into a biofilm by 18 h. Lysozyme adsorbs readily to stainless steel and maintains some of its antimicrobial activity [2], as was confirmed by our immunolabelling studies. Lysozyme thus prevented the initial attachment of cells and prevented biofilm formation. This introduces a proactive approach to biofilm control, because it provides an antimicrobial barrier against adhesion and biofilm colonization.

Self-inhibition of planktonic growth by bacteriocin like substances

A *B. flavothermus* B12-C^m biofilm inhibited the growth of B12-C^m cells on a fresh lawn of B12-C^m at specific stages in the biofilm cycle, which coincided with the peak of the biofilm cycle. This may have been a signal produced by the biofilm in response to stress and a natural part of the biofilm life cycle, resulting in the shedding of

biofilm cells from the surface. Thus, whereas normally bacteria produce bacteriocins to inhibit *related* species of bacteria, the B12-C^m biofilm cells appear to produce these bacteriocin-like inhibitory substances to inhibit the growth of planktonic cells of *their own* species. Indeed, some microbiologists give biofilms the status of a complex, multi-cultural, highly differentiated community, different from the planktonic parent cell community [28]. However, the biochemical basis of this has yet to be explained. The phenomenon may be due to the release and diffusion of small-molecular-weight inhibitors, such as lactic acid, or bacteriocin-like substances [31], or a glycolipid-rich lipid surfactant, as seen in the case of *Streptococcus thermophilus* [7]. The latter may very well be the case, as we observed that organisms from a biofilm grown on the TSA plate had a very mucoid morphology, unlike typical colonies from the planktonic cultures used to inoculate plates.

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